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##### **CHECKLIST FOR REVIEWERS**

**Title of the manuscript:** **ALGINATE-ENCAPSULATION, SHORT-TERM STORAGE AND PLANT REGENERATION FROM PROTOCORM OF *CYMBIDIUM BICOLOR* LINDL. AN EPIPHYTIC ORCHID**

**Author (s):** **G. Mahendran\*, N. Parimala Devi & V. Narmatha Bai**

# No of the manuscript: JPOP781

Deadline for the receiving of your review: 30 days after the receiving of the manuscript

**Please consider main point A and B. Please DO NOT CONTINUE TO REVIEW the manuscript if:**

**- the answer to point A.1 is YES**

**- the answer to point B is LOW.**

**A. Relevance of the paper.**

**1. *Previous publication of the material***

x No

□ Yes. What and where………………………………………………………………...

### B. Scientific and practical importance of the data

□ High

□ Adequate

□ Low

### Due to several unclear points, this question can not be answered (see comments below)

### C. Scientific quality

***1. Are the data in this manuscript new?***

x Yes

□ No. Comments:.…………………………………………………………………………

***2. Is the manuscript clearly written and well-organized?***

□ Yes

x No. Comments: The aims/objectives and intended applications of the results are not clear

***3. Are the Abstract and the Key words adequate?***

□ Yes

x No. Suggestions: there are many terms that are not used in the correct way, and contradictions lead to ambiguity regarding the overall aims (see below)

***4. Does the Introduction state the present knowledge and aim of the research?***

□ Yes

x No. Comments: The authors do not differentiate between vegetative and generative propagation. The introduction contains too much citations and should be more precisely preparing the reader to the study.

***5. Materials, methods, and study design***

□Adequate

xImprovement needed. Suggestions: The authors do not give the necessary details of the culture medium (B5), do you mean Gamborg et al. 1968??? I guess that this is not suitable for orchids. Green pods were used as starting material, how old were these seed capsules (days after pollination)? Were they derived from selfings or cross pollinations?

□Inadequate. Comments: ...........................................!

***6. Results and Discussion***

□Properly drawn with regard to methods and data

□ Should be adjusted – Suggestions: ………………………………………………….

x Insufficiently supported – Comments: Especially the results should be described and explained in more detail, the chapter results and discussion is mainly summarizing literature and sometimes it is not clear to the reader, if the own results or results obtained by others are described. The number of references should be reduced to those that are relevant.

***7. Are the tables , figures titles, and legends presented well and necessary?***

□ Yes

x Improvement needed. Suggestions: Table 1 and 2 present data with two decimals, but this accuracy is not justified by the number of measurements. Table 1: multiple protocorms??? What is meant here? Did the PLBs form multiple shoots or PLBs? Title of Table 1 not adequate (see comments below). Figure 1: Titles do not fit to the photos and all pictures seem squeezed

□ No. Comments: ........................................................................

***8. Data and statistical treatment***

□ Adequate

x Improvement needed. Comments: The authors should comment on the statistical treatment of their % data. Normally they require transformation. Did the authors check for distribution of the data and variance homogeneity?

□ Inadequate. Comments: .....................................................

***9. Have all relevant literature been cited***

□ Yes

x No. Suggestions: .Citations should be reduced to the most relevant (e.g. many of the references for synthetic seeds for other species than orchids can be omitted) and the below mentioned studies should be included.

**D. Recommendations (after corrections)**

□ The paper should be published as it is now, or with minor editorial changes

□ The paper could be published after minor revision, and need not be re-reviewed

□ The paper could be accepted after major revision according to the comments

x Rejected, may be reconsidered as new submission

#### E. If adjustments or revision is recommended

□ The writer is allowed to contact me

x I want to be anonymous

x I am not willing to review this paper again

□ I agree to review the manuscript again after the revision

Please add further comments.

This manuscript deals with *Cymbidium bicolor*, an orchid that is introduced by the authors as economically relevant/commercial. For commercial orchids propagation is nearly exclusively done by vegetative means using tissue culture techniques. However, the authors used protocorms as propagation units and proposed to encapsulate them for propagation and short-term storage. Here is the most important problem of the study, since for propagation encapsulation would be a completely unnecessary step, seeds can easily be germinated aseptically (published previously) and further propagated in vitro. For storage, in vitro plantlets can be stored under slow growth conditions, but this does not need encapsulation. In contrast, if encapsulation should be used as preparation for cryoconservation, that would be a more interesting approach, but has already been described for this species by Yam et al. 2010 (see below). Thus, in the present form, the ideas and intentions of the authors are completely unclear to me leading to my recommendation not to accept the manuscript in its present form.

Further comments that might help to improve the manuscript for a new submission:

1. Please be more careful with the terminology: In the title the authors chose plant regeneration, in the tables and text they mix germination, re-growth, viability and conversion. Germination would be the formation of plantlets from seeds, conversion means plant formation from somatic embryos/PLBs and this difference is really important! To me it is not quite clear, how the development in this system was: protocorms were embedded and incubated on medium containing cytokinins in relatively high concentrations. In consequence they did not form one shoot and root pole, but several shoots either arising from axillary buds or more likely from adventitious shoots or PLBs. This fact is not described.
2. In the introduction (lines 30 ff.) the synthetic seeds are mentioned as promising for rare hybrids, elite genotypes and sterile hybrids. This needs a vegetative propagation system, which is not given in the manuscript.
3. It would be very important to describe the morphology of the protocorms after the different storage times at 25 °C. I would assume that they grow a little bit, but not much due to missing nutrients? Furthermore, since respiration takes place their reserves probably are decreasing over time.
4. The number of plants used in the acclimatization step is missing, but urgently needed.
5. Germination is not at all defined: When was a protocorm regarded as germinated?

The following references might be interesting to cite:

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| *In vitro* plantlet regeneration from protocorms of *Dendrobium lituiflorum* Lindl. and *Cymbidium bicolor* Lindl. and their acclimatisation: effect of salts, sucrose, and banana extract.  Vyas, S. Kapai, V. Y. Kapoor, P. Guha, S. Rao, I. U.  Journal of Horticultural Science and Biotechnology; 2012. 87(5):485-492. 27 ref.  The salts present in modified Knudson C (KC) medium (1946), 2% (w/v) sucrose, and a natural banana extract (BE) [1-20% (v/v)] were studied singly, or in combination, for their effects on the regeneration of protocorms of the orchid species, *Dendrobium lituiflorum* and *Cymbidium bicolor*. The incorporation of KC salts along with higher percentages of BE [10% or 20% (v/v)] promoted protocorm development as well as shoot and root formation. The presence of 2% (w/v) sucrose in the medium further enhanced root lengths and the rooting percentage, irrespective of the presence of KC salts. Incorporation of both KC salts and 2% (w/v) sucrose was found to be obligatory, in conjunction with BE (a cost-effective natural additive) for plantlet regeneration. *In vitro*-raised plantlets of both orchid species exhibited high rates of survival under greenhouse conditions. Scanning electron microscopy revealed elliptical stomata and epicuticular granular wax deposits on their abaxial leaf surfaces after acclimatisation. |
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| 5. |  | Ex situ orchid conservation - a case study from the Singapore Botanic Gardens.  Yam, T. W. Tay, F. Ang, P. Chua, J.  Acta Horticulturae; 2010. (878):21-28. 6 ref.  Some 226 species of native orchids have been recorded in Singapore. Of these, 178 are considered to be extinct, 40 are critically endangered, one is endangered (*Bulbophyllum vaginatum*), two are vulnerable (*Vanilla griffithii*, *Bulbophyllum trifolium*), and only five are considered to be common (*Arundina gramminifolia*, *Bromheadia finlaysonianum*, *Dendrobium crumenatum*, *Eulophia graminea*, *Spathoglottis plicata*). The orchid conservation program at the Singapore Botanic Gardens aims to monitor existing species, explore ways to conserve their germplasm, and increase their number for subsequent re-introduction into appropriate habitats, including managed parks and roadsides. Thus far, we have successfully re-introduced *Grammatophyllum speciosum*, *Bulbophyllum vaginatum*, *Bulbophyllum membranaceum*, *Cymbidium finlaysonianum* and *Cymbidium bicolor*. A special designated area at the National Orchid Garden has been set up to showcase the rich diversity of native species. The Orchid Cryo-Seed Bank began several years ago and is showing some promising results. We have successfully stored seeds of several native species including *Cymbidium finlaysonianum*, *Cymbidium bicolor*, *Grammatophyllum speciosum*, *Dendrobium crumenatum*, *Spathoglottis plicata*, *Bulbophyllum vaginatum*, and *Dendrobium anosmun*. |
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| 6. |  | Conservation of the native orchids through seedling culture and reintroduction - a Singapore experience.  Yam, T. W. Chua, J. Tay, F. Ang, P.  Botanical Review; 2010. 76(2):263-274. 14 ref.  [Journal article. Conference paper]  AN: 20103185758  Singapore is located near the equator, off the southern tip of the Malay Peninsula. The whole country consists mostly of lowland. It has many interesting types of natural habitats such as primary rain forest, freshwater swamp forest, mangroves, secondary forests, shrub, grasslands, and urban parks and fields. The climate is equatorial with relatively uniform temperature and high humidity. Unfortunately, many of the natural habitats and the native orchids which thrive there have disappeared due to habitat destruction. Some 226 species of native orchids have been recorded in Singapore. However, of these 178 are considered to be extinct, and only five are common. The orchid conservation programme aims to monitor existing species, explore ways to conserve their germplasm, and increase their numbers in natural, semi-natural, and urban environments through ex-situ seedling culture and subsequent re-introduction into appropriate habitats, including roadside trees, parks and natural areas. In the first phase of the programme, we have successfully propagated and carried out experiments of re-introduction on five species of native orchids, namely, *Grammatophyllum speciosum*, *Bulbophyllum vaginatum*, *Bulbophyllum membranaceum*, *Cymbidium finlaysonianum* and *Cymbidium bicolor*. Survival percentages 8-yr after the reintroduction events ranged from 10 to 95 for *G. speciosum*, the target species of the earliest re-introduction experiments. Size of the seedlings at reintroduction, host trees, and relative humidity seemed to play significant roles in the success rate of the reintroductions.  Publisher Springer Science + Business Media, Inc (Springer)  Location of Publisher New York  Country of Publication USA |

Production of synseed for hybrid *Cymbidium* using protocorm-like bodies.

Silva, J. A. T. da

Journal of Fruit and Ornamental Plant Research; 2012. 20(2):135-146. 39 ref.

[Journal article]

AN: 20133131702

Synthetic seed were produced from protocorm-like bodies (PLBs) of hybrid *Cymbidium* Twilight Moon 'Day Light' after culture on a new medium, Teixeira *Cymbidium* (TC) medium. This new medium contained, in addition to a unique selection of macro- and micronutrients, 0.1 mg/l alpha -naphthaleneacetic acid and 0.1 mg/l kinetin, 2 g/l tryptone and 20 g/l sucrose, and was solidified with 8 g/l Bacto agar. Several explant types and sizes (intact PLBs, half-PLBs, PLB longitudinal thin cell layers) were tested. In addition, pretreatment of PLB-synseeds with 200 mM KNO3 solution, the addition of activated charcoal or coconut water to synseeds, light vs dark culture, short-term (1 month) and long-term (6 and 12 months) low-temperature (4 degrees C) storage, as well as cryostorage were also tested. All treatments resulted in less PLBs than the control treatment. Among all these treatments, only the use of TC medium or incorporation of coconut water into synseeds resulted in "germination" while low-temperature storage (1-6 months) was only possible under liquid TC. These results would allow for the short-term preservation of *Cymbidium* germplasm but not for effective cryopreservation.

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| Cryopreservation of *Cymbidium eburneum* Lindl. and *C. hookerianum* Rchb. f., two threatened and vulnerable orchids via encapsulation-dehydration.  Kiran Gogoi Suman Kumaria Pramod Tandon  In Vitro Cellular & Developmental Biology - Plant; 2013. 49(3):248-254. 37 ref.  [Journal article]  AN: 20133193008  A successful cryopreservation protocol for the long-term conservation of protocorms of two threatened and vulnerable orchids, *Cymbidium eburneum* Lindl. and *Cymbidium hookerianum* Rchb. f., was developed using encapsulation-dehydration. Protocorms were osmoprotected in liquid Murashige and Skoog medium (MS) containing 0.7 M sucrose for 20 h at 25+or-2 degrees C on a rotary shaker, and incorporated into an encapsulation matrix [consisting of 3% (*w/v*) sodium alginate and 100 mM CaCl2]. The encapsulated protocorms, which were desiccated in a laminar airflow cabinet for 6 h, were able to withstand cryostorage in liquid nitrogen. Maximum regeneration into complete plantlets (72% for *C. eburneum* and 70% for *C. hookerianum*) of the cryostored, encapsulated protocorms was obtained using MS medium containing 3% sucrose and 0.8% agar. Using this protocol of cryopreservation, long-term preservation for ex situ conservation of these two threatened orchids can be accomplished. |